Statement Under 37 CFR 1.97(e)

☐ Each item of information contained in this Information Disclosure Statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of this Information Disclosure Statement; or

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Statement Under 37 CFR 1.704(d) (Patent Term Adjustment)
Applies to original applications (other than design) filed on or after May 29, 2000

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☒ Enclosed herewith is form PTO-1449:

- ☒ Copies of the cited references (AZ11-AS12) are enclosed.
  - ☐ Copies of issued U.S. patents and published U.S. applications are not required and are not being provided.

☐ Copies of the cited references are enclosed except those entered in prior application, U.S. Application No. [ ], to which priority under 35 U.S.C. 120 is claimed. **[OPTIONAL** [The earlier application contains copies of the cited references.]

☐ The listed references were cited in the enclosed International Search Report in a counterpart foreign application.

☐ The "concise explanation" requirement (non-English references) for reference(s) [ ] under 37 CFR 1.98(a)(3) is satisfied by:

- ☐ the explanation provided on the attached sheet.
- ☐ the explanation provided in the Specification.
- ☐ submission of the enclosed International Search Report.
- ☐ submission of the enclosed English-language version of a foreign Search Report and/or foreign Office Action.
- ☐ the enclosed English language abstract.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Junming Le, Jan Vilcek, Peter Daddona, John Ghrayeb, David Knight and Scott Siegel		
Application No.:	10/774,118	Group:	1644
Filed:	February 6, 2004	Examiner:	Phillip Gambel
Confirmation No.:	8464		
For:	RECOMBINANT A2-SPECIFIC TNF-α SPECIFIC ANTIBODIES (AS AMENDED)		

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This Information Disclosure Statement is submitted:

☒ under 37 CFR 1.97(i):

Applicant requests that the IDS and cited references be placed in the application file. Applicants inadvertently failed to include these references previously. These references were acknowledged and considered by the Examiner in related applications. For example, these references were cited in the IDS filed on August 23, 2006 in USSN 11/400,787 (U.S. Patent No. 7,179,893, issued February 20, 2007), as references AU11, AV11 and AX11. The Examiner is invited to acknowledge and consider the IDS and the references.

☐ Applicant requests that the following non-published pending applications be considered:
(Affix a label or apply the stamp "Non-Published IDS Reference - Do Not Scan" to the front of each unpublished pending appl'n.)

Examiner's Initials

_____ U.S. Patent Application No. [ ], by [inventor(s)], filed [ ], Docket No.: [ ]

_____ U.S. Patent Application No. [ ], by [inventor(s)], filed [ ], Docket No.: [ ]

_____ U.S. Patent Application No. [ ], by [inventor(s)], filed [ ], Docket No.: [ ]

_____ _____
Examiner Date

☐ A copy of each above-cited application, including the current claims, is enclosed, except any application filed on or after June 30, 2003, which has been scanned into the PTO's Image File Wrapper (IFW) system and is available to the examiner.

☐ A copy of each above-cited application, including the current claims, is enclosed, except those entered in prior application, U.S. Application No. [ ], to which priority under 35 U.S.C. 120 is claimed.

The Examiner is requested to return a copy of the above list of pending applications indicating which references were considered with the next office communication.

It is requested that the information disclosed herein be made of record in this application.

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Respectfully submitted,

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Dated: April 17, 2007

PTO-1449 REPRODUCED	ATTORNEY DOCKET NO. 0975.1005-038	APPLICATION NO. 10/774,118	
SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT IN AN APPLICATION	FIRST NAMED INVENTOR Junming Le	FILING DATE February 6, 2004	
April 11, 2007	EXAMINER Phillip Gambel	CONFIRMATION NO. 8464	GROUP 1644
(Use several sheets if necessary)			

OTHER DOCUMENTS *(Including Author, Title, Date, Pertinent Pages, Etc.)*		
	AZ11	Goh, C.R., "Tumour Necrosis Factors in Clinical Practice," *Ann. Acad. Med. Singapore, 19*(2):236-239 (1990).
	AR12	Socher, S.H. *et al.*, "Antibodies Against Amino Acids 1-15 of Tumor Necrosis Factor Block its Binding to Cell-Surface Receptor," *Proc. Natl. Acad. Sci. USA, 84*:8829-8833 (1987).
	AS12	Morrison, S.L. *et al.*, "Vectors and Approaches for the Eukaryotic Expression of Antibodies and Antibody Fusion Proteins." In *Antibody Engineering, Second Edition,* C.A.K. Borrebaeck, ed. (NY: Oxford University Press), p. 291 (1995).

EXAMINER	DATE CONSIDERED

Annals, Academy of Medicine, Singapore Vol. 19 No. 2 March 1990

ONCOLOGY

Guest Editors:
C J Oon *AM, MD (Cantab), FRCP (Lond)*
S H Chan *AM, PhD, FRCPA*

CONTENTS

Tumour Necrosis Factors in Clinical Practice

C R Goh,* *MBBS (Lond), MRCP (UK)*

Abstract

The tumour necrosis factors are pleiotropic proteins which have a wide range of biological activities. Tumour necrosis factor-α or cachectin is a product of macrophages and is the principal host mediator of septic shock and the cachexia of chronic disease. A related molecule, tumour necrosis factor-β or lymphotoxin, is produced by T lymphocytes in response to antigen or mitogens. The role of the TNFs in disease processes and in therapy are reviewed. Both agents exert antiproliferative effects on certain tumour cell lines, while normal cells are resistant to these effects. In vivo, they cause haemorrhagic necrosis of certain implantable tumours in mice. Trials of tumour necrosis factor-α as an anticancer agent, either singly or in combination with interferon gamma or cytotoxic drugs, are in progress. Understanding the involvement of the tumour necrosis factors in pathological processes may lead to new therapies for endotoxic shock and cancer.

Keywords: *Cachectin, Cachexia, Cancer, Clinical practice, Lymphotoxin, Septicaemia*

Introduction

There are currently two cytokines which are known as tumour necrosis factors (TNFs), designated TNF-α and TNF-β. They are both polypeptide hormones which share a wide variety of biological activities. They are involved in a number of physiological and pathological processes, some of which are clinically important. This paper reviews some of the published data on these interesting molecules and relate them to our current understanding of their place in disease processes and in therapy.

TNF-α/CACHECTIN

TNF-α, also known as cachectin, is the better studied molecule. The reason for its dual name is because of the history of its discovery. TNF-α was used therapeutically at the end of the last century when William Coley,[1] a New York surgeon, demonstrated that injection of bacterial products derived from *Streptococcus* and *Serratia* locally into oropharyngeal tumour sites induced tumour regression. The treatment was known as Coley's toxins. In 1975, Carswell[2] showed that a soluble factor in the serum of animals treated with endotoxin was able to cause haemorrhagic necrosis of tumours and this was termed tumour necrosis factor.

Cachectin was studied by separate groups as a substance which caused severe cachexia in rabbits infected with *Trypanosoma brucei*, through the depression of lipoprotein lipase activity in fatty tissue.[3] Further studies showed that this was caused by a macrophage factor induced by lipopolysaccharide and other substances of microglial cells of the brain, have been shown to produce Beutler et al[5] noted that the N-terminal sequence of mouse cachectin was strikingly similar to that of human TNF-α that cachectin and TNF-α were found to be one and the same substance.

TNF-α is a molecule principally made by activated macrophages, though other cells of the immune system, such as lymphocytes, natural killer cells, astrocytes and microglial cells of the brain have been shown to produce it.[6] The mature protein has 157 amino-acids and is non-glycosylated (Table I). It exists both as a membrane-bound molecule on the surface of macrophages and in a free secreted form. It is produced as a prehormone with a 76 amino-acid signal peptide which is believed to act as

TABLE I: COMPARISONS OF TNF-α AND TNF-β

	TNF-α	TNF-β
Cellular origin	macrophages	T lymphocytes
Mol Wt	17,356	18,800
Mature protein	157 a.a.	171 a.a.
Prepeptide	76 a.a.	34 a.a.
Glycosylation	no	single site
Membrane-bound form	yes	not known
Free form	yes	yes

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is fully established. Glucocorticoids are potent inhibitors of TNF-α synthesis and release.[27] Their use in septic shock has only been reported to be successful if given when the patient first arrives at hospital, before any diagnosis has been made.[28] Should neutralising monoclonal antibodies to TNF-α prove to be useful in clinical practice, they will also have to be given early in the disease. What is needed is a simple bed-side test for TNF-α, which will allow early diagnosis and guide therapy. Cyclooxygenase inhibitors have been found to alleviate many of the toxic effects of TNF-α administration.[29] Clinical trials will be needed to see whether these have a role in the management of endotoxic shock.

Cachexia: TNFs are implicated in the cachexia associated with many chronic diseases, including parasitic infections,[30] some cancers[31] and AIDS.[32] Raised serum levels of TNF-α have been reported in these conditions. The source of this TNF-α is believed to be macrophages invading inflammatory tissues or autonomous production of TNF-α by neoplastic tissue. Chronic administration of TNF-α to animals is known to induce cachexia, anorexia and a catabolic state.[33] Neutralising monoclonal antibodies to TNF-α have been proposed as treatment for cachexia of chronic inflammatory and neoplastic disease but no reports of attempts at this is yet available.

Inflammation and Infection: TNF-α acts directly on the hypothalamus as a pyrogen. It can also induce the synthesis of interleukin-1 (IL-1).[34] It enhances chemotaxis of macrophages and neutrophils and increases their phagocytic and cytotoxic activity.[35] It has been implicated in autoimmune disease,[36] the acute phase of graft-versus-host disease[37] and in renal allograft rejection.[38] In rheumatoid arthritis, antibodies to TNF-α reduced IL-1 production by synovial cells. Intra-articular injection of anti-TNF-α antibodies has been suggested for therapy of rheumatoid arthritis.[39]

Serum TNF-α is raised in patients with malaria,[30] and high levels are associated with a poor prognosis in children with cerebral malaria.[40] Antibodies to TNF-α can protect mice from cerebral complications of *Plasmodium berghei* infection.[41] Their use in therapy of patients with falciparum malaria has yet to be reported.

TNFs can activate macrophages to induce killing of the schistosomula of Schistosoma mansoni,[42] and are implicated in the defense against Chlamydia infections.[43] Both TNF-α and TNF-β have been reported to have antiviral properties, either directly or through the induction of or synergy with other mediators such as interferon-gamma (IFN-γ).[44,45]

Tissue Remodelling: The physiological role of TNFs is not known. One which has been proposed for TNF-α is in the turnover and remodelling of tissues. TNF-α and IL-1 are released by macrophages during the removal of senescent proteins bearing advanced glycosylation end-product (AGE) moieties.[6] The TNF-α released can induce collagenase, proteases, reactive oxygen intermediates and arachidonic acid metabolites to help breakdown tissues, as well as growth factors which stimulate the laying down of new tissue.

TNF-β is a potent osteoclast activating factor and stimulates bone resorption in tissue culture.[46] Both IL-1 and TNF-β are implicated in the causation of the hypercalcaemia associated with epithelial carcinomas and haematological malignancies.[47] Human myeloma cells have been shown to produce TNF-β with bone resorptive activity, and infusion of TNF-β in mice leads to a rise in plasma calcium levels.[48]

Malignancy: The name TNF was given to the substance found in the serum of endotoxaemic mice that caused tumour lysis. Both TNF-α and TNF-β have cell killing and growth inhibitory effects on many tumour cell lines. But a number of cell lines of non-tumour origin are resistant to the cytotoxic and cytostatic activities of TNFs, and these cytokines may paradoxically have growth stimulatory effects on some of these lines.[49,50] Both TNF-α and TNF-β may stimulate cells to express genes which are associated with terminal differentiation.[51,52] Together these findings led to their use in anticancer therapy.

Given *in vivo*, the TNFs cause haemorrhagic necrosis of certain implantable tumours, notably the cholanthrene-induced (Meth A) sarcoma of mice. There is evidence that this haemorrhagic necrosis is mediated by the effect of TNFs on endothelial cells, causing extravasation and destruction of the centre of the tumours, while leaving an outer rim of viable tumour tissue.[53] This effect is seen both when the agents are given systemically and intratumorally. They may therefore have a place as tumour debulking agents. Other factors are necessary to bring about complete regression of the tumour, including an intact immune system.

TNF-α has been used in Phase I studies in patients with advanced cancer.[54] The regimes used include IM or IV rhTNF-α (specific activity ~ 4×10^7 units/mg protein) in doses ranging from 1–200 μg/m². Maximum tolerated doses (MTD) range from 125–800 μg/m². Overall response rates of TNF-α used alone in a variety of advanced cancers is of the order of 3.3%, very similar to rates obtained with single agents such as 5-fluorouracil or carboplatin.[55] Moderate to severe dose-related toxic side-effects were observed, including fever, chills, anorexia, nausea, hypotension, myelosuppression and hepatotoxicity. Cyclooxygenase inhibitors, such as indomethacin and ibuprofen, have been reported to alleviate many of the toxic side effects of TNF-α[29] and experimental work indicates that the cytotoxic effect is not affected. Their

concomitant use in TNF-α therapy alleviates some of the dose-limiting side-effects, and allows an increase in MTD.

Because results of Phase I trials of TNF-α as a single agent have so far been disappointing, interest has shifted to its use in combination therapy. IFN-γ and TNF-α show synergistic antiproliferative effects against a wide range of human tumour cells.[49,56] Phase I trials of TNF-α in combination with IFN-γ have established maximum tolerated doses of 150 $\mu g/m^2$/day for five days for each agent.[57] Side-effects of fever, chills and fatigue were almost universal. Nausea and vomiting, myalgia and hepatotoxicity were common, especially at higher dosage. Both hypo- and hypertension have been reported.[58] Phase II studies are currently in progress. Combination therapy with other cototoxic chemotherapeutic agents is also being studied. Work in mice has shown that rh TNF-α is able to augment the effect of certain cytotoxic drugs, in particular, cyclophosphamide, if given 48 hours earlier.[59]

Future Therapy: TNF-β is currently not easily available because of initial difficulty in expression of the recombinant protein. It is hoped that when clinical grade material becomes available, trials with TNF-β will be conducted to see if this has a place in cancer therapy. Several groups are working to make improved versions of TNF-α and TNF-β in the hope of obtaining a mutant molecule with improved efficacy and decreased toxic effects. Biological effects of some TNF-α mutants have been reported, and muteins of increased cytotoxicity have been evaluated *in vitro*.[60,61] Protein engineering of a hybrid IFN-γ/TNF-β molecule to exploit the synergistic effect of IFN-γ with TNF-β has also been achieved.[62] Studies of the biological effects of such molecules *in vivo* are awaited with interest.

Apart from cancer therapy, molecular engineering may also enable a molecule to be produced which can antagonise the effects of TNF-α, a competitive antagonist for the TNF receptor. This will have application in the management of endotoxic shock. Already, neutralising monoclonal antibodies to TNF-α have been studied in the treatment of septic shock. As receptors for cytokines are cloned and studied, future therapies may involve the administration of solublised TNF receptors to absorb circulating TNF-α in septic shock and cachexia.

Conclusion

The tumour necrosis factors are pleiotropic molecules which have a large repertoire of biological activities. Understanding the involvement of these molecules in many physiological and pathological processes will lead to the use of new therapies to combat many age-old diseases.

Acknowledgement

I thank Dr A G Porter for useful advice and criticisms on this manuscript.

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Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 8829–8833, December 1987
Biochemistry

Antibodies against amino acids 1–15 of tumor necrosis factor block its binding to cell-surface receptor

(cachectin/synthetic peptide/lipoprotein lipase/cytolysis/endotoxin)

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Communicated by Edward M. Scolnick, August 21, 1987

ABSTRACT Human tumor necrosis factor (hTNF) mediates a variety of biologic activities, which are dependent on the attachment of hTNF to cell-surface receptors. To identify regions of the hTNF protein involved in binding hTNF to its receptor, we prepared five synthetic peptides [hTNF-(1–15), hTNF-(1–31), hTNF-(65–79), hTNF-(98–111), and hTNF-(124–141)] and two hydroxylamine cleavage fragments [hTNF-(1–39) and hTNF-(40–157)] of hTNF. The hTNF-synthetic peptides and hTNF fragments were tested in hTNF receptor binding assays and in two biologic assays: cytolysis of tumor cells and suppression of lipoprotein lipase in adipocytes. Neither the synthetic peptides nor hTNF fragments were active agonists or antagonists in these assays. The synthetic peptides were also conjugated to thyroglobulin, and peptide-specific antisera were raised. All five peptide–thyroglobulin conjugates induced antibody responses to the immunizing peptide and to hTNF. Each antiserum was tested for antagonist activity in hTNF binding assays. Only antisera raised against hTNF-(1–15) or hTNF-(1–31) and antisera against whole hTNF blocked binding. IgGs purified from these three antisera also block hTNF-induced cytolysis and lipoprotein lipase suppression. We conclude that antibodies that recognize the N-terminus of hTNF block the attachment of hTNF to its cellular receptor and inhibit the biologic effects of hTNF.

Tumor necrosis factor (TNF) is a cytokine secreted by activated macrophages. Carswell *et al.* (1) originally showed that TNF can promote the lysis of tumor cells *in vitro* and *in vivo*. Beutler *et al.* (2) showed that TNF was identical to another macrophage-derived protein termed cachectin. More recently, TNF has been shown to mediate a variety of biologic effects including the following: endotoxin-induced shock (3), suppression of lipoprotein lipase (LPL) activity in preadipocytes (2), stimulation of collagenase activity and prostaglandin E_2 production by synovial cells (4), enhanced proliferation of fibroblasts *in vitro* (5), stimulation of bone resorption (6), stimulation of interleukin 1 production (7), and induction of cachexia in nude mice (8). Human TNF (hTNF) cDNAs and genomic DNA clones have been isolated and sequenced (9, 10). Using these cloned genes several investigators have expressed recombinant hTNF protein and validated the diversity of biologic activities attributed to TNF (11).

TNF-specific cell-surface receptors are present on several types of cells (12, 13). The binding of TNF to these receptors is believed necessary for induction of the biologic effects of TNF. Therefore, it should be possible to inhibit the biologic activities of TNF by blocking the association of TNF with its cellular receptors. We examined the deduced amino acid sequence of hTNF and selected regions of the protein that might be involved in receptor–ligand interactions. To evaluate the importance of these regions in mediating the interaction of hTNF with its receptor we used two strategies. (*i*) Synthetic peptides were prepared based on the amino acid sequence in these regions; and the peptides were tested for the ability to inhibit cellular binding of hTNF. Fragments of hTNF were also isolated following chemical cleavage of recombinant hTNF and tested as inhibitors of hTNF binding. (*ii*) The synthetic peptides were used to produce rabbit antisera that recognize individual peptides and bind to hTNF; the antisera were tested for their abilities to inhibit hTNF binding and to block the biologic effects of hTNF *in vitro*.

We report that antisera raised against peptides representing the N-terminus of hTNF block the binding of hTNF to its cell-surface receptors. Concomitant with the inhibition of hTNF attachment to its receptor, these antisera block the biologic activities of hTNF.

METHODS

Synthesis of Peptides and Preparation of Peptide–Thyroglobulin Conjugates. Five hTNF peptides (Fig. 1) were synthesized using the solid-phase method (14). hTNF-(1–31) was prepared in our laboratory. The other peptides were obtained from Bachem Fine Chemicals (Torrance, CA). To facilitate the generation of immunoreagents, a tyrosine residue was added to the carboxyl termini of hTNF-(1–15) and hTNF-(98–111) and to the amino terminus of hTNF-(65–79). The cysteines at residues 69 and 101 were replaced with α-aminobutyric acid to minimize structural ambiguities during subsequent conjugation reactions.

The peptides were conjugated to thyroglobulin as a carrier protein for immunization purposes. Three methods of conjugation were used: amino group coupling using glutaraldehyde (15), carboxyl-activated coupling using carbodimide (16), and tyrosine coupling with bis-diazotized benzidine (17). hTNF-(1–31) was conjugated using only the glutaraldehyde method. All other peptides were conjugated by all three methods. Conjugation reactions were terminated by dialysis, with multiple changes of the dialysis medium; no further purification of the product was done.

Purification and Cleavage of TNF. Recombinant hTNF was purified from *Escherichia coli* bearing a plasmid with a hTNF gene (18). The specific activity of the purified hTNF was $1 - 5 \times 10^7$ cytolytic units per mg and was greater than 95% pure by $NaDodSO_4$/PAGE analysis.

Inspection of the amino acid sequence of hTNF (11) revealed a single Asn-Gly sequence at residues 39–40. Asn-Gly sequences are susceptible to cleavage with hydroxylamine. Using the conditions of Bornstein and Balian (19),

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Abbreviations: TNF, tumor necrosis factor; LPL, lipoprotein lipase; hTNF, human tumor necrosis factor.

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a sample of recombinant hTNF was digested with hydroxylamine. The digest was fractionated on a Sephadex G-100 gel filtration column (1.6 × 100 cm) in 20% (vol/vol) HOAc/H_2O. hTNF-(1–39) that was >95% pure was isolated from this chromatography. Purity was assessed by amino acid composition (20) and sequencing (21, 22). A second peak was shown by NaDodSO$_4$/PAGE to consist of a mixture of hTNF-(40–157) (major species) and undigested hTNF (minor species). This material was purified by gel filtration chromatography on a Superose-12 column (Pharmacia, HR 10/30) installed in a Pharmacia Fast Protein Liquid Chromatography unit. The chromatogram was developed in a pH 8.5 buffer containing 0.2 M Tris hydrochloride and 4 M guanidine hydrochloride. Following dialysis of the various peaks, homogeneous (NaDodSO$_4$/PAGE) hTNF-(40–157) was identified and collected.

Production of Antisera and IgG Purification. New Zealand White rabbits were immunized with 250 μg of each peptide–thyroglobulin conjugate in complete Freund's adjuvant by intradermal injections. Beginning 4 weeks later subcutaneous injections with the conjugates in incomplete Freund's adjuvant were repeated every 2 weeks for 8 weeks. Hyperimmune antisera to recombinant hTNF and to the conjugate of hTNF-(1–31)/thyroglobulin were produced by intramuscular injection of 100 μg of hTNF and 250 μg of hTNF-(1–31)/thyroglobulin in complete Freund's adjuvant; this was followed 3 weeks later by a similar injection in incomplete Freund's adjuvant and then intravenous injection of the antigens in aqueous solution at 5 and 7 weeks after the first immunization. Sera were collected biweekly beginning 3 weeks after the first injection of antigen. IgG fractions were purified from selected sera as described by Ey *et al.* (23).

ELISA. Microtiter plates (96-well) were coated with 0.1 μg of peptide or hTNF at 4°C overnight. After the plates were washed, 0.1 ml of dilutions of rabbit serum or IgG fractions were added, and the plates were incubated at 37°C for 0.5 hr. The plates were washed and incubated for 0.5 hr at 37°C with 0.1 ml of goat anti-rabbit IgG–alkaline phosphatase conjugate (Kirkegaard and Perry, Gaithersburg, MD). Plates were washed, and 0.1 ml of 4-nitrophenyl phosphate (Sigma) was added. After a 0.5-hr incubation at 37°C, the plates were read at 405 nm with a Bio-Tek (Burlington, VT) EL310 platereader.

RIA. Reaction mixtures of 0.3 ml contained 0.05 ml of serum or IgG fraction, 0.1 ml of 90 pM ^{125}I-hTNF and 0.1 ml containing 15% yeast extract and 15% skim milk. Samples were incubated at 4°C for 12–14 hr, and then 0.1 ml of protein A-Sepharose 4B (75 mg/ml) was added. After an incubation for 1.5 hr at 4°C, the tubes were centrifuged, and the pellets were washed three times in 0.5 ml of phosphate-buffered saline. Radioactivity was determined using an Abbott Autologic γ counter.

Cytolysis Assay. Cytolysis was done by modification of the procedure of Ruff and Gifford (24). L929 cells (obtained from American Type Culture Collection) and HeLa R19 cells (obtained from R. Colonno, this laboratory) were seeded in 96-well plates at 30,000 and 40,000 cells per well, respectively, in 0.1 ml of growth medium and incubated overnight at 37°C in 5% CO_2. hTNF and hTNF peptides with or without antibodies and actinomycin D (0.15 μg) were added in 0.05 ml of media containing 30 mM Hepes, pH 7.4. Plates were incubated for 18 hr at 37°C in 5% CO_2. Cells were stained with 0.5% crystal violet in 20% methanol, washed, and solubilized with 0.5% NaDodSO$_4$. Absorbance at 540 nm was measured using a Bio-Tek EL310 platereader.

Lipoprotein Lipase Assay. 3T3-L1 cells were obtained from American Type Culture Collection. Differentiated 3T3-L1 cells (25) in 96-well plates were exposed to hTNF and hTNF peptides with or without antibodies for 12–18 hr at 37°C. After treatment, the medium was withdrawn from each well, and 0.1 ml of medium containing heparin (10 units per ml) and insulin (50 ng/ml) was added for 1 hr at 37°C. The LPL assays were done as described by Kawakami *et al.* (26).

TNF Iodination and Binding Studies. Recombinant hTNF was iodinated using the method of Baglioni *et al.* (27). The recovery of purified ^{125}I-hTNF was determined using a sandwich ELISA (8). ^{125}I-hTNF had a specific activity of 40–80 μCi/μg (1 Ci = 37 GBq), and the recovery of biologic activity based on cytolysis of L929 cells was 90–95% of unlabeled hTNF. Binding assays were done using HeLa R19 cells seeded in 24-well plates at 2.5 × 10^5 cells per well 18–24 hr before use, or 3T3-L1 cells prepared as described. Cells were placed at 4°C and washed with 1 ml of medium; then 0.25 ml of medium containing ^{125}I-hTNF and 25 mM Hepes, pH7.4, was added to each well. The plates were incubated at 4°C for 4 hr with gentle rocking. The cells were washed four times with 0.5 ml of medium, solubilized with 0.5 ml of 1M NaOH, transferred to vials, and counted in a γ counter.

RESULTS

The primary amino acid sequence of hTNF was analyzed using several techniques. Hydropathicity was examined using the parameters of Hopp and Woods (28) and Kyte and Doolittle (29). Secondary structural features including alpha

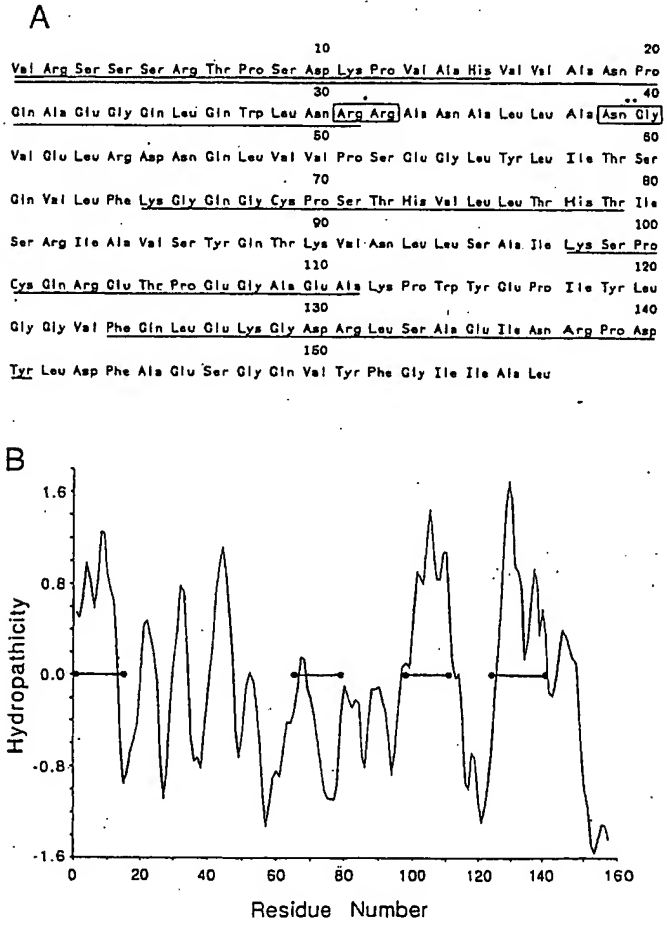

FIG. 1. Structural analysis of hTNF. (*A*) Amino acid sequence of hTNF. *, Arg-Arg sequence (boxed) at residues 31–32, putative proteolytic processing site. **, Asn-Gly sequence (boxed) at residues 39–40, site of hydroxylamine cleavage. Underlined sequences represent the peptides that were synthetically prepared. (*B*) Hydropathicity plot for hTNF, using the parameters of Hopp and Woods (28). Heavy lines in the center of the plot indicate the peptides that were originally chosen for synthesis based on hydropathicity, secondary structural features, or predicted immunogenicity of amino acid content.

helices, turns, and amphipathic helices were predicted using the methods of Chou and Fasman (30) and Schiffer and Edmundson (31). Putative antigenic sites were selected in accord with the findings of Welling *et al.* (32). Fig. 1 depicts three regions of high hydrophilicity. Three peptide sequences [hTNF-(1–15), hTNF-(98–111), and hTNF-(124–141)] were selected from these regions based on the assumption that hydrophilic regions will be located on the surface of hTNF and therefore may be involved in receptor–ligand interactions. A fourth peptide sequence [hTNF-(65–79)] was selected despite being located in a hydrophobic region of hTNF. This peptide contains residues that are likely to cause a bend or turn in the structure of hTNF and contains amino acids that probably are immunogenic.

Two other features of the primary amino acid sequence influenced our strategy. An Arg-Arg site exists at residues 31–32. Several prohormones are processed into their mature form by cleavage at similar dibasic sites (33). Therefore, we selected the sequence hTNF-(1–31) for analysis on the assumption that this peptide might be a biologically active cleavage product of hTNF. An Asn-Gly site exists at residues 39–40. Asn-Gly linkages are uniquely sensitive to cleavage by hydroxylamine. We took advantage of this Asn-Gly site to split recombinant hTNF into two fragments in our attempt to localize the binding domain of hTNF to one of these fragments.

The synthetic hTNF peptides and hTNF fragments were tested for activity as agonists or antagonists of hTNF in whole cell binding assays, cytolysis assays, and LPL suppression assays. The synthetic peptides were tested at concentrations up to 10^6 times the effective dose required to produce a half-maximal response (ED_{50}) with hTNF (data not shown). None of these peptides exhibited activity in our assays. Similarly, neither of the hTNF fragments exhibited activity in these assays when tested individually, or in combination at concentrations up to 200 times the ED_{50} of hTNF.

Because the synthetic peptides did not behave as agonists or antagonists of hTNF in direct assays, an indirect immunologic approach was used to assess the importance of these amino acid residues in receptor binding. Rabbits were immunized with synthetic peptide–thyroglobulin conjugates or with hTNF. Sera were initially screened in an ELISA to assess their binding to immobilized peptides and hTNF. All antipeptide antisera bound to their homologous peptide and to recombinant hTNF (Table 1). However, variations were observed in the ELISA titers of antisera against the immunizing peptide compared with the ELISA titer of the same antisera against hTNF. To characterize the binding properties of the antisera to hTNF under more physiologic conditions, RIAs were done. Antisera raised against hTNF-(1–15), hTNF-(1–31), and recombinant hTNF bound the majority of ^{125}I-hTNF in RIAs. Antisera to hTNF-(65–79), hTNF-(98–111), and hTNF-(124–141) bound 10% or less of the ^{125}I-hTNF (Table 1). Similarly, antisera obtained from rabbits immunized with hTNF-(1–15), hTNF-(1–31), and recombinant hTNF blocked the specific binding of ^{125}I-hTNF to the surface of HeLa R19 cells. Antisera obtained with hTNF-(65–79), hTNF-(98–111), and hTNF-(124–141) did not alter hTNF binding (Table 1). Comparable results were obtained in whole cell binding assays using 3T3-L1, L929, or ME-180 cells (obtained from American Type Culture Collection).

Having identified reagents that inhibit the interaction of hTNF with its receptor, we asked if these antisera would affect hTNF-induced cytolysis or LPL suppression in cell culture. These assays were complicated by the fact that preimmune rabbit sera induced cytolysis of HeLa R19 and L929 cells and inhibited LPL activity. To circumvent this problem the IgG fractions of each antisera were purified on protein A-Sepharose columns, and the purified IgGs were analyzed in these assays. As seen in Table 2, IgG isolated from preimmune rabbit serum did not induce cytolysis or LPL suppression in our assays. IgGs obtained from antisera that blocked the binding of hTNF to its cellular receptors on HeLa R19 cells [anti-hTNF-(1–15), anti-hTNF-(1–31), and anti-hTNF] inhibited hTNF-induced cytolysis. Similarly, antibodies that blocked the binding of hTNF to 3T3-L1 cells inhibited the ability of hTNF to suppress LPL activity (Table 3). In both the cytolysis and LPL suppression assays IgGs obtained from antisera directed against hTNF-(65–79), hTNF-(98–111), and hTNF-(124–141) failed to inhibit the effects of hTNF (Tables 2 and 3).

The inhibitory activities of anti-hTNF-(1–15) IgG and anti-hTNF-(1–31) IgG can be blocked by preincubation of the IgG fractions with either hTNF-(1–15) or hTNF (1–31) peptides before the addition of the antibodies to the hTNF bioassays. Preincubation with hTNF-(65–79), hTNF-(98–111), or hTNF-(124–141) does not block the neutralizing

Table 1. Binding of antipeptide and anti-hTNF antisera to hTNF in ELISA, RIA, and radioreceptor assays

Antigen		ELISA titer*		Radioreceptor assay, % inhibition	RIA, % input cpm bound
Peptide	Coupling agent	Homologous peptide	hTNF		
hTNF-(1–15)	EDC	16,000	64,000	93	52
	BDB	128,000	512,000	100	77
	GA	128,000	1,024,000	77	22
hTNF-(1–31)	GA	1,024,000	1,024,000	96	71
hTNF-(65–79)	EDC	25,600	800	0	5
	BDB	12,800	400	3	4
	GA	51,200	3,200	1	2
hTNF-(98–111)	EDC	1,000	16,000	4	8
	BDB	16,000	512,000	0	7
	GA	64,000	256,000	6	8
hTNF-(124–141)	EDC	32,000	512,000	2	10
	BDB	256,000	2,048,000	4	7
	GA	512,000	1,024,000	1	7
hTNF	—	—	256,000	100	96
None (preimmune serum)				3	1

Coupling agents include glutaraldehyde (GA), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), and bis-diazotized benzidine (BDB).
*ELISA titers are expressed as the reciprocal of the sample dilution at endpoint.

Table 2. Effects of IgG antibodies to hTNF peptides and hTNF on binding and cytolysis in HeLa R19 cells

Antigen				
Peptide	Coupling agent	ELISA titer*	Inhibition of ^{125}I-hTNF binding,† IC_{50} (%)	Inhibition of cytolysis,‡ IC_{50} (%)
hTNF-(1–15)	BDB	2,000,000	0.0048	0.049
hTNF-(1–15)	GA	4,000,000	0.58	1.10
hTNF-(1–31)	GA	4,000,000	0.11	1.45
hTNF-(65–79)	GA	16,000	>10.0	>10.0
hTNF-(98–111)	GA	500,000	>10.0	>10.0
hTNF-(124–141)	GA	4,000,000	>10.0	>10.0
hTNF	None	2,000,000	0.0024	0.027
None (preimmune)		<1,000	NE	NE

Coupling agents include glutaraldehyde (GA) and bis-diazotized benzidine (BDB). NE, no effect.
*ELISA titers to hTNF are expressed as the reciprocal of the sample dilution at endpoint.
†Purified IgG fractions were added to the binding mixture containing 125 pM ^{125}I-hTNF and incubated for 1.5 hr at room temperature before addition to cultures of HeLa R19 cells.
‡Purified IgG fractions were incubated with 1.3 nM hTNF for 2 hr before addition with actinomycin D to HeLa R19 cells.

activity of anti-hTNF-(1–15) IgG or anti-hTNF-(1–31) IgG. We also attempted to block the neutralizing activity of anti-hTNF IgG by preincubation with the five synthetic peptides either alone or in combination. The synthetic peptides failed to reduce the neutralizing activity of anti-hTNF IgG. This result prompted us to examine the ability of anti-hTNF to bind the synthetic peptides in both the ELISA and RIA. As seen in Table 4 anti-hTNF does bind efficiently to hTNF-(1–15) and hTNF-(1–31) peptides. Because these peptides do bind but cannot block the neutralizing activity of anti-hTNF IgG, the anti-hTNF antisera must recognize other neutralizing determinants on hTNF in addition to those found on the synthetic peptides hTNF-(1–15) and hTNF-(1–31).

DISCUSSION

The amino acid sequence of recombinant hTNF consists of 157 residues that form a 17,000-kDa polypeptide (11). These monomers spontaneously aggregate to form noncovalently linked multimeric species under physiologic conditions (34, 35). Biologically active recombinant hTNF exists as a trimeric species (34). It is unclear whether monomeric hTNF is also biologically active. The receptor binding domain and contact points for the attachment of hTNF to its cellular receptors have not been identified. However, the c-terminal 16 amino acids of a related cytokine, lymphotoxin, are required for biologic activity (36).

We find that antibodies directed against the N-terminus of hTNF block the association of radiolabeled hTNF with its cell-surface receptors. Both anti-hTNF-(1–31) and anti-hTNF-(1–15) antisera block hTNF binding. Antibodies directed against three other regions of hTNF do not inhibit receptor binding. Recently, Creasey and coworkers also reported that the N-terminus of hTNF is necessary for receptor binding and biologic activity (37, 38). These investigators prepared a series of N-terminal deletion mutants of hTNF. Structurally modified hTNF proteins were expressed and isolated from these mutants and subsequently tested for biologic activity. Truncated proteins missing either four, seven, or eight N-terminal amino acids exhibited full biologic activity in tumor cell cytotoxicity assays. However, removal of the N-terminal 10 amino acids from hTNF significantly decreased receptor binding and cytotoxicity. Because the N-terminal eight amino acids of hTNF are not required for receptor recognition and because anti-hTNF-(1–15) antibodies block receptor binding, the critical amino acids in this region may involve residues 9–15.

Anti-hTNF-(1–15) antibodies may inhibit binding by masking amino acid residues that serve as contact points between hTNF and its receptor. If these residues are contact points, they should be located on the external surface of hTNF. The hydropathicity plot of the hTNF in Fig. 1 indicates that residues 1–15 form a hydrophilic domain. Therefore, the N-terminus is likely to be found on the solvent-exposed surface of the molecule. The fact that anti-hTNF-(1–15) antibodies recognize and precipitate hTNF in RIAs also suggests that the N-terminus is accessible to interaction with other proteins in solution. Alternatively, the N-terminal

Table 3. Effects of IgG antibodies to hTNF peptides and hTNF on ^{125}I-hTNF binding and LPL activity in 3T3-L1 cells

Antigen			
Peptide	Coupling agent	Inhibition of ^{125}I-hTNF binding,* %	Inhibition of TNF-induced LPL suppression,* %
hTNF-(1–15)	BDB	94	102
hTNF-(1–15)	GA	81	84
hTNF-(1–31)	GA	95	97
hTNF-(65–79)	GA	2	0
hTNF-(98–111)	GA	5	5
hTNF-(124–141)	GA	3	7
hTNF	None	98	96
None (preimmune)		6	2

Coupling agents include bis-diazotized benzidine (BDB) and glutaraldehyde (GA).
*Purified IgG fractions were added at 1% to the binding mixture containing 80 pM ^{125}I-hTNF and to the LPL mixture containing 80 pM unlabeled hTNF and then incubated for 1.5 hr at room temperature before addition to 3T3-L1 cells.

Table 4. Binding of hTNF antisera to hTNF peptides and hTNF fragments in ELISA and RIAs

hTNF peptide/fragment	ELISA titer*	RIA†
1–15	64,000	92
1–31	128,000	92
65–79	<100	100
98–111	<100	94
124–141	2,000	94
1–39	128,000	NT
40–157	64,000	NT
hTNF	256,000	3
None (preimmune)		100

NT, not tested.
*ELISA titers are expressed as the reciprocal of the serum dilution at endpoint.
†0.05 ml of 1:250 dilution of anti-hTNF serum was incubated in a 0.20-ml volume for 1 hr at 37°C with the individual hTNF peptides or hTNF at 100 nM before addition of 100 μl of 120 pM ^{125}I-hTNF. Data are expressed as % cpm bound.

amino acid residues of hTNF may contribute to the three-dimensional configuration or the multimeric structure of hTNF required for biologic activity. Anti-hTNF-(1–15) antibodies might disrupt this configuration or structure resulting in biologically inactive hTNF. It is also possible that anti-hTNF-(1–15) antibodies block the binding of hTNF to its receptor via nonspecific steric interference. Antisera produced against whole hTNF also inhibit hTNF binding. This inhibitory activity cannot be removed with preincubation of the anti-hTNF antisera with synthetic hTNF peptide 1–15. Therefore, other regions of hTNF besides the N-terminus are likely to participate in receptor recognition and/or structural stabilization of biologically active hTNF.

hTNF induces a broad spectrum of biologic activities. Some of these activities can have severe deleterious effects in animals (3, 8). Our data indicates that the biologic effects of hTNF can be inhibited by antisera that block the attachment of hTNF to its cell-surface receptors. Therefore, hTNF receptor binding antagonists may be useful therapeutics in clinical situations characterized by inappropriate or excessive hTNF production.

We thank Dr. Michael Rosenblatt for thoughtful discussions and Ms. Rita Taylor for assistance in preparing the manuscript.

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Antibody Engineering

Second Edition

Edited by

Carl A. K. Borrebaeck

New York Oxford
Oxford University Press
1995

Oxford University Press

Oxford New York Toronto
Delhi Bombay Calcutta Madras Karachi
Kuala Lumpur Singapore Hong Kong Tokyo
Nairobi Dar es Salaam Cape Town
Melbourne Auckland Madrid

and associated companies in
Berlin Ibadan

Published by Oxford University Press, Inc.,
200 Madison Avenue, New York, New York 10016

Oxford is a registered trademark of Oxford University Press

Library of Congress Cataloging-in-Publication Data

Antibody engineering / edited by Carl A.K. Borrebaeck. — 2nd ed.
p. cm. (Breakthroughs in molecular biology)
Includes bibliographical references and index.
ISBN 0-19-509150-7
1. Immunoglobulins—Biotechnology.
2. Protein engineering.
I. Borrebaeck, Carl A. K., 1948–
II. Series.
TP248.65.I49A57 1995
616.07'93 dc20 94-25809

1 3 5 7 9 8 6 9 2

Printed in the United States of America
on acid-free paper

tates are analyzed on 12% Tris-glycine gels following treatment with 0.15 M 2-mercaptoethanol.

After running the gels until the bromophenol blue is approximately 1 cm from the bottom of the gel (100 to 150 mA/gel for phosphate gels, 150 V for tris-glycine gels), the gels are treated for 10 minutes with staining solution (50% methanol, 7% acetic acid, 0.02% Coomassie blue), and then destained using 7% acetic acid and 5% methanol. Gels may be left in destain overnight. After the gels are dried onto filter paper (Whatman 3MM) they are exposed to X-ray film. The exposure time required depends on the amount of Ig being produced. If enhancement of radioactivity is required, rinse gels once in distilled water, and then add 1 M salicylate and incubated for 30 min prior to drying the gel. Exposure must now be at −70°C.

Things to Consider

The analysis described is designed to determine if the heavy and light chains produced by the transfectoma are of the expected sizes and are correctly assembled and secreted. Appropriate molecular weight markers must be included in the gel for comparison; frequently, well-characterized myeloma or transfectoma proteins provide convenient standards. The assembly patterns and polymerization state of the transfectoma antibodies are determined by SDS-PAGE without prior treatment with reducing agents. Examination of the cytoplasmic proteins shows the intermediates in Ig assembly. Most antibodies are assembled using either the pathway $H + L \rightarrow HL \rightarrow H_2L_2$ or $H + H \rightarrow H_2 \rightarrow H_2 \rightarrow H_2L \rightarrow H_2L_2$; the pathway utilized is usually determined by the isotype of the heavy chain.

Generally IgGs are secreted into the serum as H_2L_2, while IgA and IgM form higher polymers. If a large amount of polymers are present, use of 4% Acrylamide-phosphate gels can be useful for the resolution of the polymeric forms. Light chain not assembled with heavy chain is sometimes secreted, but as a general rule heavy chains will not be secreted without an accompanying light chain. Human IgG4 secretes H-L half-molecules; a mutation in its hinge has been observed to lead to the production of only fully assembled IgG4.[28]

Acknowledgments

Work in the laboratory is supported by grants CA 16858 and AI 29470 from the National Institutes of Health, and by grants IM-550 and IM-603 from the American Cancer Society. S.-U. Shin is supported in part by NINDS training grant 1Tew-NSO7356. M.J. Coloma is a Centocor Fellow.